Eidelman, A., Cohen, C., Navarro-Castilla, Á., Filler, S., Gutiérrez, R., Bar-Shira, E., Shachar, N., Garrido, M., Halle, S., Romach, Y., Barja, I., Tasker, S., Harrus, S., Friedman, A., & Hawlena, H. (2019). The dynamics between limited-term and lifelong coinfecting bacterial parasites in wild rodent hosts. *Journal of Experimental Biology*, *222*. https://doi.org/10.1242/jeb.203562

The dynamics between limited-term and lifelong coinfecting bacterial parasites in wild rodent hosts

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A running title

Multifaceted coinfection

Keywords:

Coinfection, concomitant infections, mixed infections, multiple parasites, within-host competition, within-host facilitation.

A summary statement

Coinfection with both limited-term and lifelong bacteria induces physiological and behavioral changes in a rodent host that may make these bacteria's interaction dynamic over time.

Abstract

Interactions between coinfecting parasites[1] may take various forms, either direct or indirect, facilitative or competitive, and may be mediated by either bottom-up or top-down mechanisms. While each form of interaction lead to different evolutionary and ecological outcomes, it is challenging to tease them apart throughout the infection period. To establish the first step towards a mechanistic understanding of the interactions between coinfecting limited-term bacterial parasites and lifelong bacterial parasites, we studied the coinfection of *Bartonella* sp. (limited-term) and *Mycoplasma* sp. (lifelong), which commonly co-occur in wild rodents. We infected *Bartonella* and *Mycoplasma*-free rodents with each species, and simultaneously with both, and quantified the infection dynamics and host responses. *Bartonella* benefited from the interaction; its infection load decreased more slowly in coinfected rodents than in rodents infected with *Bartonella* alone. There were no indications for bottom-up effects, but coinfected rodents experienced various changes, depending on the infection period, in their body mass, stress levels, and activity pattern, which may further affect bacterial replication and transmission. Interestingly, the infection dynamics and changes in the average coinfected rodent traits were more similar to the chronic effects of *Mycoplasma* infection, whereas coinfection uniquely impaired the host's physiological and behavioral stability. These results suggest that parasites with distinct life history strategies may interact, and their interaction may be asymmetric, non-additive, multifaceted, and dynamic through time. Since multiple, sometimes contrasting, forms of interactions are simultaneously at play and their relative importance alternates throughout the course of infection, the overall outcome may change under different ecological conditions.

Introduction

Due to the ubiquity of coinfecting parasites[1] and their potential impact on disease outcomes, biodiversity, and host-parasite coevolution, there is a growing interest in revealing the mechanisms underlying the interactions between them (McArdle et al., 2018; Pedersen and Fenton, 2007; Rynkiewicz et al., 2015; Seppala and Jokela, 2016; Telfer et al., 2010). Parasitic organisms may chemically or mechanically affect the growth, reproduction, or transmission of their coinfecting counterparts (e.g., Grube et al., 2011; Hawlena et al., 2010). Coinfecting parasites may also interact indirectly through bottom-up mechanisms such as exploitative competition or by producing substances that aid in resource exploitation (Poulin, 2001; Ramiro et al., 2016; Wale et al., 2017; West and Buckling, 2003) or through top-down mechanisms. The latter mechanisms may be mediated by the host behavioral or immunological responses (Reviewed in Cox, 2001; Dianne et al., 2010; Mabbott, 2018; Supali et al., 2010) or through the damage caused to the host (e.g., Gleichsner et al., 2018; Mendez-Lozano et al., 2003; Yin et al., 2017).

Teasing apart the different above interaction mechanisms is important since they can lead to different evolutionary outcomes (Mideo, 2009). For example, exploitation competition is predicted to lead to selection, either for divergence in resource use between competitors or increased virulence. In contrast, interference competition is expected to select for lower virulence, and top-down mechanisms are predicted to select for adaptations such as immunomodulation to host responses (reviewed by Mideo, 2009). Different interaction forms can also lead to different ecological and epidemiological outcomes. For instance, knowledge on the underlying mechanisms can explain patterns of parasite diversity (Bashey, 2015; Zele et al., 2018), as well as spatial and temporal variability in parasite-parasite, and host-parasite interactions in natural communities (Jolles et al., 2008; Pedersen and Fenton, 2007; Penczykowski et al., 2016). However, teasing apart interaction mechanisms among coinfecting parasites is not trivial and demands simultaneous exploration of multiple, reciprocal host and parasite responses throughout the infection period (Mideo, 2009; Zele et al., 2018). This challenge might explain the scarcity of mechanistic studies on interactions between unrelated coinfecting parasitic organisms despite the pervasiveness of their co-occurrence (Karvonen et al., 2019). Such studies, above all, require knowledge of and practice with various organisms, differing in their life histories and methods of host

[1] "Parasite" is used throughout the article in its broad definition as an organism that lives in or on an organism of another species (its host) and benefits by consuming parts of the host resources or components. It damages the host but is rarely lethal in the short term. This term includes herein viruses, bacteria, protozoa, helminths, ectoparasites, and other blood-sucking organisms.

exploitations. Moreover, since often facilitation and competition are regarded as different ecological paths (e.g., Mideo, 2009; Zele et al., 2018), the potential involvement of both components in the interaction has been overlooked (but see; Cattadori et al., 2014; Kamiya et al., 2018).

Rodents coinfected with *Bartonella* and hemotropic *Mycoplasma* bacteria (hemoplasmas) in the Negev Desert sand dunes, Israel, constitute a convenient system in which to tease apart the mechanisms of interactions between unrelated coinfecting parasitic organisms throughout the infection period, including facilitative and competitive components. On one hand, these are widespread and prevalent coinfecting genera among wild and domestic mammals. Both employ the host red blood cells (RBCs) as targeted cells and multiply within the vascular system, providing various opportunities to interact and coevolve. On the other hand, the two bacterial genera are phylogenetically distant and have different ecological niches [*Mycoplasma* spp. (hemoplasmas) parasitize the RBC outer membrane while *Bartonella* spp. penetrate the RBCs], transmission routes, and persistency levels (Barker and Tasker, 2013; Cohen et al., 2018; Gutiérrez et al., 2015; Harms and Dehio, 2012). Importantly, the species from these two genera that coinfect Gerbillina rodents demonstrate two ends of the invasion-persistency continuum. '*Candidatus* Bartonella krasnovii' bacteria (Gutiérrez et al., 2018) are easily transmitted between rodents through fleas, leading to limited-term bacteremia (i.e., up to six months; Morick et al., 2011; 2013), whereas *Mycoplasma haemomuris*-like infections are characterized by low transmission rates (mainly via host-to-host contact) and lifelong infections (Cohen et al., 2018). Thus, the exploration of within-rodent *Bartonella-Mycoplasma* interactions is expected to provide insights into the mechanisms underlying coinfection between parasitic organisms with different life history strategies that share the same resources. Finally, co-occurrence analyses indicate positive *Bartonella-Mycoplasma* associations in blood sampled from wild *Gerbillus andersoni* captured in the southeast Negev (Kedem et al., 2014) and negative associations in blood sampled from *G. andersoni* captured in the northwest Negev (Cohen et al., 2015a). Thus, it is likely that *Bartonella-Mycoplasma* interactions include both positive and negative components, but their balance changes under different ecological conditions.

To establish the first step towards a mechanistic understanding of *Bartonella-Mycoplasma* interactions, we compared the infection dynamics and the changes in the rodent host's physiological and behavioral variables in response to infection with '*Ca.* B. krasnovii' (designated herein as *Bartonella*) and *Mycoplasma haemomuris*-like bacteria (designated herein as *Mycoplasma*) alone and to simultaneous coinfection with these two bacterial species

under laboratory conditions. The infection dynamics was used to deduce, from the parasite perspective, the outcome of the interaction under laboratory conditions. In particular, lower loads of bacteria in coinfection compared to a single infection would support competition, while higher loads would support facilitation. The quantification of changes in rodent variables was used to highlight candidate interaction mechanisms, where RBC loss in coinfected rodents (measured by packed blood cell volume) would suggest exploitative competition, higher antibodies would suggest immune-mediated competition, and the opposite effects would suggest the parallel facilitative interactions. Since the main transmission route of *M. haemomuris*-like bacteria is assumed to be through host-to-host aggressiveness (Cohen et al., 2018), we also predicted that to increase transmission, *Mycoplasma*, like other parasitic organisms (Hughes and Libersat, 2019; Moore, 2002), would induce greater rodent movement (i.e., a higher activity duration, frequency, or both). Following this logic, from the *Mycoplasma* perspective, increased activity of coinfected rodents would support facilitation, whereas reduced rodent activity would support competition. Finally, to highlight candidate interaction mechanisms mediated through host damage, we compared the rodent body mass gain, body temperature, physiological stress levels, activity pattern, and the daily variability in these variables, between singly infected and coinfected rodents. However, since it is not yet clear whether the damage to the host measured by these variables benefits or harms each bacterial species, we could not use these measurements to locate our predictions on the competition-facilitation continuum.

Materials and Methods

Study animals

Rodents: All *Gerbillus andersoni* that were used in the study were born and raised in the laboratory and were PCR-negative for *Mycoplasma* and *Bartonella* bacteria. The rodents were at least seven months old, non-reproductive, with an average body mass of 38.79 ± 5.22 g (± standard error; SE) and 36.11 ± 4.01 g for males and females, respectively. Rodents were maintained individually in 20×30 cm^2 plastic cages with a 1-cm layer of autoclaved sand as substrate, in an animal facility with an air temperature of 25 ± 1^0C and a photoperiod of 12 h dark: 12 h light. They were provided daily with millet seeds *ad libitum* and 13.3 ±1.4 g alfalfa as a water source according to Hawlena et al. (2007).

Bacteria: *Mycoplasma* and *Bartonella* are the most dominant genera in *G. andersoni* blood (Cohen et al., 2015b; Gavish et al., 2014). Regardless of the geographical region,

rodent community composition, and rodent species, the *Mycoplasma* found in all blood samples belong to a single cluster, which is closely related to, but distinguishable from, *M. haemomuris*, and is therefore termed *M. haemomuris*-like (Kedem et al., 2014). In contrast, diverse *Bartonella* species may infect and coinfect *G. andersoni* rodents, but commonly, individuals are found infected with '*Ca.* B. krasnovii' (Gutiérrez et al., 2018). Thus, by infecting *G. andersoni* rodents with *M. haemomuris*-like bacteria, '*Ca.* B. krasnovii', or both, we emulated the most common infection and coinfection scenarios in nature.

Experimental design

Forty *G. andersoni* rodents (1:1 male to female ratio) were subjected randomly to four groups, each composed of ten individuals: (i) a control group inoculated with 500 µl of phosphate-buffered saline (PBS), (ii) rodents infected with 1.0×10^4 *M. haemomuris*-like bacteria in 500 µl PBS, (iii) rodents infected with 2.1×10^8 '*Ca.* B. krasnovii' in 500 µl PBS, and (iv) rodents coinfected with 1.0×10^4 *M. haemomuris*-like bacteria and 2.1×10^8 '*Ca.* B. krasnovii' in 500 µl PBS. To assess the infection dynamics of the two bacterial species in rodents from the three infection groups, we bled them every 10 days following the inoculation day until day 122 post-inoculation. In parallel, we quantified the host variables over a period ranging from one to four days during each of the main infection stages, namely (i) before the inoculation (days −15–0), (ii) an infection peak (days 11–24), (iii) a sharp decrease in bacteremia (days 30–42), (iv) a slow decrease in bacteremia (with only a few *Bartonella* bacteria; days 42–65), and (v) when *Mycoplasma* loads reached a plateau, as *Mycoplasma* stabilized on a low bacterial load and *Bartonella* could not be detected (days 87–122) (Fig. 1 and Table 1). The sampling days were chosen to minimize the interference among measurements. Accordingly, the behavioral measurements (see below) were always recorded at least four days after bleeding and before fecal collection, and we omitted the behavioral records during feeding and temperature measurements, including 30 min after these activities. Similarly, fecal collection was conducted at least two days post-bleeding, and the temperature measurements were recorded at least four days post-bleeding.

Bacterial inoculation and quantification

M. haemomuris-like bacteria, similarly to other hemotropic *Mycoplasma* species, are uncultivable organisms (Tasker et al., 2003); therefore, rodents were subcutaneously inoculated with blood from *Mycoplasma*-positive *G. andersoni*, preserved in 20% DMSO

(Sigma-Aldrich, Buchs, Switzerland) and stored at −80°C. After thawing, the inoculum was diluted with PBS to reach a concentration of 1.0×10^4 cells in 500 µl (confirmed by qPCR). This concentration was chosen since it minimizes the blood volume needed for the inoculation (58 ± 6.3 µl of required donor blood; mean and SE, respectively) to allow 100% infection success (Cohen, Eidelman, and Hawlena, unpublished data). Regardless, the transmission success, the infection load, and the timing of peak infection of *M. haemomuris*-like bacteria are dose-independent (Cohen et al., 2018). To prepare a *Bartonella* inoculum, a wild-type '*Ca.* B. krasnovii' was maintained in the laboratory, and after six passages on chocolate agar plates, the colonies were diluted in PBS to reach a concentration of 2.1×10^8 colony-forming unit (CFU) in 500 µl (based on colony counting and confirmed by qPCR). This concentration was chosen since it is the minimum number of bacteria required for 100% success of infection with this species (Cohen, Eidelman, and Hawlena, unpublished data).

Despite the low volume of blood subcutaneously injected to rodents in the *Mycoplasma* and coinfected infection groups (~12% of the inoculum and ~2% of their blood volume), we intended to exclude the possibility that the blood itself generated the differences between these groups and the control and *Bartonella* groups, which were injected with only PBS. Accordingly, at the end of the experiment, we simultaneously subcutaneously injected six *G. andersoni* rodents with an average of 50 ± 13 (± SE) µl of blood from bacteria-free rodents in a total volume of 200 µl solution (~25% of the inoculum and ~2% of their blood) and six *G. andersoni* with 200 µl PBS. We then compared the physiological and behavioral changes of these two rodent groups over 122 days.

To assess the bacterial loads in the rodent blood over the 122 experimental days, in every bleeding event, 100–200 µl of blood was collected from the retro-orbital sinus of each individual by sterile capillaries immersed in 0.15% EDTA and stored in EDTA tubes at −20°C until further molecular analyses. The bleeding was conducted under local anesthesia (Localin, Fischer Pharmaceutical Labs, Tel Aviv, Israel). DNA was extracted from blood samples using a MoBio Bacteremia DNA Isolation Kit, following the manufacturer's instructions (Cohen et al., 2015b). In each extraction session, a negative control was included, in which all of the reagents were added to PBS instead of the blood.

Quantification of the bacterial copy numbers (designated as bacterial loads) was performed by a real-time quantitative PCR (qPCR) (CFX Connect™ System, Hercules, California, USA), using 2 x qPCRbio Fast Qpcr Probe Blue Mix, Hi-ROX (PCR Biosystems).

For *Mycoplasma*, the following were added to the master mix: 200 nM of the 16S rRNA gene forward primer (MhmI 458F) CGCCGAATACTGCTCGTC and 200 nM of the 16S rRNA gene reverse primer (MhmI 590R) TCAAGCCTAAGCGTCAATAGC ,100 nM of probe (Mhm 634P) FAM/AACACCAGA/Zen/GGCGAAGGCGAAA/3IABkFQ, 4 µM of $MgCl_2$ and 5 µL of DNA in a total volume of 25 µL. PCR conditions were 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 30 s at 60°C.

For *Bartonella*, the following were added to the master mix; 300 nM of the *gltA* gene forward primer GGATTTGGTCACCGAGTCTATAAA, 300 nM of *gltA* gene reverse primer AAGAAGCGGATCGTCTTGAATAT, 200 nM of probe CCACGTGCAAAAATCATGCAAAAAACCTGTCA, and 2 µl of DNA in a total volume of 20 µL (PrimerDesign Ltd., Chandlers Ford, UK). PCR conditions were 3 min at 95°C followed by 35 cycles of 10 s at 95°C and 30 s at 60°C.

We ran 2–3 replications per sample, and in each run, we included a positive control (a sample with a known bacterial concentration) and a negative control (ultrapure water).

To estimate the absolute copy numbers and validate the repeatability, efficiency, and sensitivity of the reactions, in each run, we added a 10-fold serial dilution (i.e., standard curve ranged from 10^2–10^7 copies per reaction) of previously sequenced plasmids containing either the 16S rRNA gene of *M. haemomuris*-like bacteria or the *gltA* gene of '*Ca.* B. krasnovii '. To avoid overestimation of absolute numbers by plasmid standards (Kim et al., 2014), the standard curve was calibrated by Digital Droplet PCR (ddPCR), which separates each sample into a large number of partitions and then runs the PCR reaction in each partition individually, allowing a direct count of the nucleic acid molecules. To assess the specificity of the *Bartonella* qPCR, we ran, in addition, 20 DNA samples extracted from blood collected from the *Mycoplasma*-infected rodents at an infection peak (days 11 and 21) as well as 18 samples of control individuals, using the *gltA* primers. Similarly, to assess the specificity of the *Mycoplasma* qPCR, we ran 40 DNA samples extracted from blood collected from the *Bartonella*-infected rodents at an infection peak (days 11 and 21) and at a period of sharp decrease in bacteremia (days 30 and 42), as well as 40 samples of control individuals, using the 16S rRNA gene primers.

Physiological and behavioral measurements

Packed RBC volume (PCV): After collecting the blood through a 0.15% EDTA-coated capillary into EDTA tubes (see bacterial inoculation and quantification), we left the

remaining blood in the capillary, sealed it on one side, and centrifuged it at 13.3 RPM for 12 min. Then, we measured the packed cell volume.

Bartonella specific IgG: We used anti-*Bartonella* IgG as a measure of the specific immune response. We prepared heat-killed '*Ca*. B. krasnovii' antigens by heating bacterial cells in PBS at 56°C for 1 h. We then determined the *Bartonella*-specific IgG antibody levels in rodent sera sampled by ELISA, following Bar-Shira et al. (2003). Briefly, immuneplates (Nunc, ThermoFisher Scientific, Waltham, MA USA) were coated with *Bartonella* antigens at a concentration of 5×10^8 cells/ml in a carbonate-bicarbonate coating buffer with pH 9.6. Coated plates were placed in a humidified chamber at 4°C overnight. Plates were blocked for 2 h at 37^0C, using 0.5 % skim milk (BD, Difco, Sparks, MD, USA) in PBS. Then, serum samples diluted 1:1000 in a blocking solution were added, and plates were incubated at 37^0C for 1 h. *Bartonella*-specific antibodies were detected using HRP-conjugated rabbit anti-gerbil IgG (GeneTex, Irvine, CA, USA), where TMB (Kirkegaard and PerryLaboratories, Gaithersburg, MD, USA) was used as a substrate. Color development was terminated by TMB stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Optical absorbance was determined at 450 nm, using an EL $\times$ 808^{TM} Absorbance Microplate Reader (Bio Tek,Winooski, VT, USA). To enable the comparison of samples among runs, all readings were normalized to *Bartonella*-positive and *Bartonella*-negative serum samples, which were run in all the ELISA plates. This allowed us to ensure that the *Bartonella*-specific reading obtained for each of the tested samples reflected the differences in the immune response raised against *Bartonella* and were not dependent on the experimental conditions. Unfortunately, due to the uncultivable nature of *Mycoplasma*, we could not run parallel *Mycoplasma*-specific ELISA assays. However, the similar dynamics of these bacteria in single and coinfected rodents (see Results section below) suggest that immunity to *Mycoplasma* does not play a key role in mediating the interactions between the two bacterial species.

Body mass: Rodents were weighed in the mornings of the bleeding days before bleeding.

Surface body temperature at rest: One week before the first measurement of the pre-inoculation period (day −14), we subcutaneously implanted calibrated temperature transponders (Bio Medic Data Systems, IPTT-300) in all rodents. Daily measurements were recorded in the morning, 90 min after first light, when rodents were resting, by holding a

transponder reader above the host cage without opening the cage or disturbing the host rest. These measurements are designated hereafter as body temperatures.

Physiological stress levels: We quantified the fecal corticosterone metabolites (FCMs). The fecal glucocorticoid levels are indicators for stress levels in vertebrates, including rodents (Navarro-Castilla et al., 2017; Sanchez-Gonzalez et al., 2018; Touma et al., 2004). For the fecal collection, one hour after dark, each rodent was placed in a clean plastic cage with a bowl of seeds and a wire mesh floor above a paper, allowing the feces to fall to and be collected from the paper. After 12 h, we collected the fresh feces, saved them in −20°C until further analysis, and returned the rodents to their original cage. Corticosterone quantification was done by enzyme immunoassay (EIA), following Navarro-Castilla et al. (2018). Briefly, fecal samples were primarily dried in a heater (3 h, 90ºC). Then, 0.05 g of the dry feces was mixed with 500 µl of 100% methanol and 500 µl of phosphate buffer, and shaken for 16 h in a multivortex. Later, samples were centrifuged (2500 g, 15 min), and fecal extracts were stored at −20°C until analysis. For the quantification of FCM levels, we used a commercial corticosterone EIA (DEMEDITEC Diagnostics GmbH, D-24145 Kiel, Germany) previously used in rodent species (Abelson et al., 2016; Navarro-Castilla et al., 2017). This EIA was specifically validated for *G. andersoni* through an ACTH challenge test, and the laboratory EIA performance was verified by parallelism, accuracy, and precision tests.

Activity pattern: We placed a motion detector (model Swan Quad; Crow Group, Airport City, Israel) 40 cm above each cage, which continuously counted the rodent movements (every 20 s, the activity was either coded as “1” or “0”, for movement or no movement, respectively) into data loggers. We then extracted the data and calculated the mean average time of a movement bout, designated as activity duration, and the movement frequency (i.e., number of movement bouts), designated as activity frequency in the light (diurnal) and dark (nocturnal) hours.

The handling protocol was approved by the Committee for the Ethical Care and Use of Animals in Experiments of Ben-Gurion University of the Negev (# IL-59-09-2015).

Statistical analysis

To compare between the bacterial load (dependent variable) in singly infected and coinfected rodents (independent variable), we performed, for each infection period and bacterial species, a generalized linear model (GLM). For each of the rodent’s immunological, physiological, and behavioral measurements, we calculated first the means and SEs of the days included in each infection period per individual rodent. The means and SEs were then used to evaluate

the changes in the trait values and trait variability, respectively, by calculating the difference between the values measured in each infection period and the values of the pre-inoculation period (designated as the change in the measured variable). We then tested with GLMs whether sex, treatment, and the interaction between them (independent variables) could significantly explain the variability in these observed changes (dependent variables). Since we were mainly interested in the comparisons between the single-infection groups and the control group and between the coinfection group and the three other treatment groups and not between the two single-infection groups, we also ran specific planned comparisons of least square means between the groups of interest. In both the bacterial and rodent analyses, we first tested a full factorial design, and when the sex × treatment interaction was not significant, we repeated the analysis, excluding the effect of sex.

To exclude the possibility that the blood injection itself generated the differences between the *Mycoplasma*-infected and coinfected groups and the other two groups that were injected with only PBS, for each significant difference found between those groups, we ran parallel statistical comparisons between the clean blood- and PBS-inoculated rodents to confirm that we did not get the same between-group differences. All analyses were performed using STATISTICA 12 software (StatSoft Inc., USA).

Results

Apart from one coinfected individual that became sick during the experiment and thereby was excluded from all following analyses, the remaining 39 rodents were monitored throughout the 122 experimental days. All individuals, singly and coinfected, became successfully bacteremic at day 11 post-inoculation (Fig. 1). The qPCR assays showed high specificity. First, the DNA extracts of the control individuals showed only low values (mean estimated loads and SE of 2 ± 2 and 3 ± 3 per 1 µl of DNA for the *gltA* and 16S rRNA primers, respectively). Second, the cross-reaction rate between *Bartonella* and *Mycoplasma* was low, with $2 \pm 2\%$ cross-reaction for the *gltA* primers and $2 \pm 1\%$ cross-reaction for the 16S rRNA primers.

Individuals infected with *Bartonella* showed a typical limited-term infection pattern, reaching a peak bacterial load by day 20 and a sharp bacteremic decline from day 21 onwards to a complete elimination by day 87 post-inoculation in the blood of any individual (Fig. 1A). All individuals remained *Bartonella*-negative during the remaining experimental days. Individuals infected with *Mycoplasma* also showed a reduction in bacterial loads after day 21

of the infection, but their blood remained persistently infected throughout the 122 experimental days (Fig. 1B). The *Mycoplasma* dynamics in the blood of coinfected rodents also showed a chronic persistent pattern and was not significantly different from the dynamics in the single bacterial species infected rodents (Fig. 1C, Table 1). In contrast, while all coinfected individuals, similarly to the *Bartonella* (only)-infected individuals, showed no indications for the presence of *Bartonella* in their blood from day 87 onwards, the *Bartonella* reduction rate was slower in the former, reaching significantly greater loads at the sharp decrease in their bacteremia period (Table 1, Fig. 2A). The same qualitative results of significant differences between the *Bartonella*-infected and coinfected groups and non-significant differences between the *Mycoplasma*-infected and the coinfected group were obtained after we subtracted from the observed bacterial loads the maximum false positive values (as estimated through the additional cross-reaction and negative control evaluations; data not shown).

A trend of a lower increase in *Bartonella*-specific antibodies (IgG) in peak infection in coinfected rodents compared to singly infected rodents was noticed, but this trend was not significant ($p = 0.2$).

Due to technical problems, we could not analyze the stress hormones of seven individuals or measure the body temperature of ten individuals, decreasing the sample size of the stress hormone analysis to 32 (nine control, seven coinfected, eight *Bartonella*-infected, and eight *Mycoplasma*-infected rodents) and of the body temperature to 29 (seven control, seven coinfected, eight *Bartonella*-infected, and seven *Mycoplasma*-infected rodents). From all the physiological and behavioral traits measured, only the changes in physiological stress levels and in the variability in the diurnal activity frequency (DAF) were significant at the whole GLM level (Table 1: significant treatment × sex interaction for stress levels in plateau and significant treatment for DAF in sharp decrease). In particular, in male rodents, coinfected individuals had significantly higher FCM levels than both *Bartonella*-infected and control individuals. The effect of *Mycoplasma* on DAF is described below.

The planned comparisons between groups further revealed significant differences in various infection periods (Table 1; Figs. 1–2). The *Bartonella*-infected rodents gained significantly less body mass than the coinfected individuals at the period of slow decrease in bacteremia (Fig. 2C). The *Mycoplasma*-infected rodents exhibited a decrease in body temperature during the "*Mycoplasma* plateau" period (Fig. 2D) and a higher increase in the diurnal variability of activity duration during the peak infection period (Fig. 2I) compared to

the control individuals. *Mycoplasma*-infected rodents also had a stronger decrease in the variability of diurnal activity frequency during all periods of infections compared to the coinfected and control individuals (Fig. 2J). In addition to the above differences between coinfected rodents and the other groups, they showed a decrease in the variability of nocturnal activity duration during the peak infection period (Fig. 2H) and a higher increase in physiological stress levels during the period of slow decrease in bacteremia (Fig. 2E) compared to the control individuals. They also demonstrated a higher increase in the variability of body temperature during the period of sharp decrease in bacteremia compared to *Bartonella*-infected individuals (Fig. 2G), and a higher variability in body mass during the period of slow decrease in bacteremia compared to *Mycoplasma*-infected rodents (Fig. 2F). All the other planned comparisons were not statistically significant (Table 1).

Discussion

Much emphasis has previously been placed on the documentation of within-host interactions and the exploration of their effects on epidemiological patterns, and the evolution of virulence and host resistance (reviewed in Hawlena and Ben-Ami, 2015; Pedersen and Fenton, 2007; Tollenaere et al., 2016). These effects are likely to be affected by the mechanism underlying parasite interactions. Through a long-term experimental quantification of the infection and host variable dynamics, we demonstrated that when limited-term and lifelong infecting parasitic organisms meet in a host, the outcome of their interaction is multifaceted. It is dependent on the balance among multiple, sometimes contrasting, subtle top-down mechanisms, making the overall outcome highly dependent on the ecological conditions. Below we discuss the results in light of the research challenges addressed in the introduction and their potential implications for natural communities.

Teasing apart the mechanisms underlying the interaction between distant parasitic organisms

We showed indications that even distant coinfecting bacteria with different life history strategies may interact. Coinfected rodents showed a slower decline in *Bartonella* load than rodents infected with *Bartonella* alone. This facilitative effect may be translated into higher transmission rates for coinfected rodents in nature. The packed RBC volume analysis suggests that the interactions are not direct (Table 1). Although the differences between the antibodies produced by coinfected and by rodents infected with *Bartonella* alone were not significant, due to the high within-group variability in this measurement, we cannot reject the

hypothesis that this facilitative effect is mediated by decreased specific immunity against *Bartonella*. Our results also suggest that the interactions between the two bacteria may be mediated by host damage, as the behavioral pattern of coinfected rodents was significantly different from rodents infected with each bacterial species alone, and they showed a higher physiological stress response and body mass gain (Figs. 1–2).

These indications for *Bartonella-Mycoplasma* interactions, together with recent evidence in other host-parasite systems (e.g., Ben-Ami et al., 2011; Ezenwa and Jolles, 2011; Graham, 2008), suggest that interactions between unrelated coinfecting parasitic organisms may be important determinants of host fitness, and of host-parasite population dynamics and coevolution (Ezenwa and Jolles, 2011; Karvonen et al., 2019). They also suggest that multiple top-down effects may operate simultaneously.

The effect of *Bartonella-Mycoplasma* coinfection on the host seems to be asymmetric. Coinfection under laboratory conditions benefited mainly *Bartonella* and resulted in a host-parasite dynamics that mostly resembles the long-term dynamics of infection with *Mycoplasma* alone—that is, similar effects on average host traits to those caused by *Mycoplasma* (Figs. 2D–E), a slower reduction in bacterial loads (Fig. 2A), and less damage to the host (in terms of body mass gain; Fig. 2C) compared to infection with *Bartonella* alone. These changes might have been the result of behavioral (e.g., change in activity pattern), physiological (e.g., elevation of stress hormones), and molecular mechanisms (not tested in this study) induced by *Mycoplasma* to provoke a tolerant response in the rodent host. The chronic-like coinfection effect observed here is consistent with conceptual model predictions for helminth-microparasite coinfection (Ezenwa and Jolles, 2011). The model suggests that for acute or limited-term infections, which are expected to end with either the elimination of the parasite or by host death, coinfection with a lifelong infecting parasite may alter host recovery, mortality, or both. Accordingly, for those limited-term infections such as *Bartonella* that cause only mild damage to the host, the main predicted effect of coinfection is the deceleration of their clearance (Ezenwa and Jolles, 2011). Such chronic-like effects may have a facilitative effect on the number of secondary infections that are produced in the population of a limited-term infecting parasite (Ezenwa and Jolles, 2011).

Our results also suggest that the effect of *Bartonella-Mycoplasma* coinfection is not additive. In particular, the results indicate that coinfection destabilized host physiological and behavioral responses—an effect that was not demonstrated in any of the infections with each bacterial species alone (Figs. 2F, G, J). Future studies on coinfection dynamics and mechanisms between other limited-term and lifelong infecting parasites are required to

462 determine whether the dominant effect of the lifelong parasites and the induction of non-
463 additive instability is typical for such interactions.
464 Taken together, our results, along with the experimental results on coinfection with
465 parasites with different transmission strategies, target host tissues, virulence levels, and
466 different host specificity levels, suggest that interactions between phylogenetically distant
467 parasitic organisms are mostly underlined by asymmetrical top-down mechanisms (Ben-Ami
468 et al., 2011; Duncan et al., 2015; Fellous and Koella, 2009; Vojvodic et al., 2012; Yin et al.,
469 2017; but see Graham, 2008 for a bottom-up example). This is in comparison to closely
470 related parasites, which more likely to interact through interference and bottom-up
471 mechanisms (Hawlena et al., 2012; Ramiro et al., 2016; Wale et al., 2017). However, this
472 hypothesis should be systematically tested.
473
474 ***Considering simultaneously positive and negative aspects of within-host interactions***
475 ***throughout the infection period***
476 The fact that most of the observed differences between groups were revealed only by the
477 planned comparison analyses suggests that the impacts of the single infections and
478 coinfections are subtle relative to the variability in the measured traits and may be obscured
479 by the irrelevant comparisons. These subtle effects, along with the high natural infection and
480 coinfection rates (Cohen et al., 2015a; Kedem et al., 2014), support a long coevolution
481 between these parasitic bacteria, as well as between each of the bacterial species and the
482 rodent hosts.
483 It is under this long coevolution scenario that a simultaneous exploration of multiple host
484 and parasite reciprocal responses throughout the infection period is most important for
485 understanding coinfection interactions in natural communities. First, in contrast to our
486 expectations and to evidence from other coinfection studies (e.g., Bell et al., 2006; Graham,
487 2008), the effect of coinfection on host physiological and behavioral variables persisted even
488 at or after *Bartonella* clearance (Figs. 1 and 2C, E, F, J). This prolonged effect suggests that
489 in nature, the signature of previous coinfections should be considered. Second, our data
490 suggest that multiple forms of interactions are at play, with the relative importance of each
491 one alternating throughout the course of infection (Fig. 1). Temporal changes in the
492 interaction mechanisms were also found in a malaria-rodent system, where competition
493 between strains occurred around the peak of acute infection possibly through resource
494 exploitation and an immune-mediated competitive suppression, which occurred only towards
495 the end of the acute phase of the infection (Raberg et al., 2006). Such temporal dynamics in

the relative importance of alternative mechanisms may explain why interactions between microparasites in rodents can be facilitative when infections are new, but competitive when chronic (Telfer et al., 2010). More generally, this temporal dynamics may provide the key for understanding the role that the sequence and timing of coinfection play in determining coinfection outcomes (Karvonen et al., 2019).

Whether each of the observed effects of coinfection on host variables results in facilitation or competition between the parasites would ultimately require measuring the transmission of each parasite to new hosts, to ascertain whether the net direction of the interaction is positive or negative (Ezenwa and Jolles, 2011). The increased instability in the coinfected rodent behavior and physiology is consistent with the expectations for coinfection effects, as multiple infections may disrupt the balance of energy input, use, and output within a host (Rynkiewicz et al., 2015) and is likely to negatively affect both parasites (Cao and Goodrich-Blair, 2017). However, their increased body mass gain, for example, may have either positive or negative effects on the parasites, depending on the link between body mass, immune response, and resource availability (Hawlena et al., 2005). Regardless of the effect of each change in coinfected rodent traits on the parasites, the fact that some of them are conflicting (e.g., a higher body mass gain and higher stress levels) suggests that facilitative and competitive components act simultaneously. Similarly, a simultaneous infection of rabbits with two helminth species increased the density, but decreased the fecundity, of one parasite, while having the opposite effect on the other (Cattadori et al., 2014).

In such natural communities, the outcome of the interaction may depend on the specific ecological conditions, determining the balance between the multiple positive and negative components (Ezenwa and Jolles, 2011; Fellous and Koella, 2010). For example, the improved body condition of *G. andersoni* rodents (Brand and Abramsky, 1987) and lower interspecific competition (Cohen et al., 2015a; Kedem et al., 2014) that they face in the northwest Negev, compared to their conspecifics in the southeast Negev, may explain why we found, in the former region, indications for competition (Cohen et al., 2015a) and, in the latter, indications for facilitation (Kedem et al., 2014). In particular, it is possible that when rodents are under improved conditions, the stress and instability induced by coinfection are more influential, leading to parasite competition, but when their conditions are poor, the reduced immune response and increased body mass gain associated with coinfection are more pronounced, leading to facilitation. A similar pattern was found in African buffalo, coinfected with a gastrointestinal nematode and the bacterium, *Mycobacterium bovis*, where facilitative interactions, mediated by trade-off in immune response, were detected only during the dry

season when resources were more scarce (Jolles et al., 2008). Such an interplay between positive and negative effects may underlie the spatial and temporal variability in within-host interactions in nature (Callaway, 2007; Jolles et al., 2008; Pedersen and Fenton, 2007; Penczykowski et al., 2016).

Conclusions

The major contribution of this study is the manipulation and exploration of a natural assembly of phylogenetically distant parasitic organisms to illustrate the dynamic nature of coinfection. To this end, the results encourage a holistic investigation of the within-host ecology of coinfection, including the simultaneous quantification of multiple parasite and host responses and the consideration of the host's trait variability along with the host's mean traits throughout the infection period. Additional studies on the coinfection dynamics of parasites with different life history strategies are encouraged for a better understanding of its unique behavior compared to the dynamics of conspecific parasites. Regarding *Bartonella-Mycoplasma* coinfection, future studies are encouraged to make comparisons between (i) the transmission rate of singly infecting and coinfecting parasites to new hosts at different times throughout the infection period, (ii) simultaneous and sequential coinfection dynamics (Karvonen et al., 2018), and (iii) parasite interactions under different ecological conditions (e.g., in various host species or hosts with different physiological states).

Acknowledgments

We thank N. Kronfeld-Schor, M. Einav, Y. Shani, O. Altstein, N. Azran, and A. Tsairi for valuable help during this study, and B. Rosental, M. Segoli, and Y. Shafran for helpful discussions on early versions of the manuscript. The analyses of physiological stress were partially supported by the Etho-Physiology Laboratory (Head of Laboratory, Dra. Isabel Barja), Universidad Autónoma de Madrid. This is publication number X of the Mitrani Department of Desert Ecology.

Competing interests

No competing interests declared

Funding

This work was supported by the Israel Science Foundation [1391/15 to Hadas Hawlena and 688/17 to Shimon Harrus]. C.C. was sponsored by the short-term postdoctoral fellowship of Ben-Gurion University of the Negev (BGU), and M.G. was sponsored by The Kreitman School of Advanced Graduate Studies (BGU) and the Blaustein Center for Scientific Cooperation (Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev). S.F. holds a PhD studentship at Bristol Veterinary School that is sponsored primarily by the Langford Trust, but also Zoetis Animal Health.

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Table 1. A summary of the statistical results. The upper row for each dependent variable indicates the experimental days of the measurements, the row below indicates the ANOVA's F statistics for either the "treatment × sex" interaction (when it was significant, indicated by "ξ") or "treatment" effect (when there was no indication for a "treatment × sex" interaction). Contrast estimates were tested in all cases, but only the significant comparisons are mentioned in the bottom row of each cell.

	Dependent variable	Before	Peak	Sharp decrease	Slow decrease	Myc. plateau
Changes in trait mean	Log Bart. load F statistic	NA	11, 21 0.2	31, 42 *7	50, 61 NA (mostly zeros)	87, 100, 111 NA (all zeros)
	Log Myc. load F statistic	NA	11, 21 0.3	31, 42 0.013	50, 61 0.6	87, 100, 111 0.013
	Bartonella specific IgG F statistic	-14 NA	11, 21 2	42 0.6	42, 63 2	100 0.0086
	Packed RBC volume. F statistic	-14 NA	11, 21 0.9	42 0.3	42, 52 0.4	87, 111 0.4
	Body mass F statistic	0 , -14 NA	11, 21 0.7	31, 42 1	42, 52 2 *Coin. > Bart.	100, 111 1
	Body temperature F statistic	-6, -5, -4, 0 NA	17-19, 21 0.3	25-38 0.5	49-52 2	101, 119, 122 2 *Myc < Co.
	FCM levels F statistic	-4 NA	24 0.4	NA	65 2 *Coin. > Co.	118 *ξ3 M: *Coin. >.Bart. *Coin. > Co.
	Log NAD F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.9	48-51 2	108-111 0.1
	DAD F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2	35-38 1	48-51 0.7	108-111 0.3
	NAF F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.4	48-51 0.3	108-111 0.1
	DAF F statistic	-10, -9, -8, -7 NA	15-16, 19-20 0.3	35-38 0.5	48-51 0.8	108-111 0.1
Changes in trait variability	Body mass F statistic	0, -14 NA	11, 21 1	31, 42 1	42, 52 1 *Coin. > Myc.	100, 111 0.2
	Body temperature F statistic	-6, -5, -4, 0 NA	17-19, 21 0.5	25-38 2 *Coin.> Bart.	49-52 0.8	101, 119, 122 0.029
	Log NAD F statistic	-11, -10, -9, -8 NA	15-16, 19-20 2 *Coin. < Co.	35-38 0.8	48-51 2	108-111 0.4
	DAD F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2 *Myc. > Co.	35-38 2 §*Myc. < Co.	48-51 0.085	108-111 0.4
	NAF F statistic	-11, -10, -9, -8 NA	15-16, 19-20 1	35-38 0.9	48-51 1	108-111 0.16
	DAF F statistic	-10, -9, -8, -7 NA	15-16, 19-20 2 *Coin. > Myc.	35-38 *4 **Coin. > Myc. ***Co. > Myc.	48-51 2 *Coin. > Myc.	108-111 2 *Coin. > Myc. *Co. > Myc.

Bart.: *Bartonella*; Myc: *Mycoplasma*; NAD: nocturnal activity duration; DAD: diurnal activity duration; NAF: nocturnal activity frequency; DAF: diurnal activity frequency; Co: control; Coin: coinfection; M: males. *$p < 0.05$; **$p < 0.01$, ***$p < 0.005$. § The same trend was found between blood-injected and PBS-injected controls; thus, this effect was omitted.

Figure legends

Fig 1. Bacterial dynamics and significant changes in rodent variables throughout the experimental period. Mean ± SE of *Bartonella* (light grey) and *Mycoplasma* (dark grey) load in 1 µl of DNA extracted from the blood of either *Bartonella*-infected (A), *Mycoplasma*-infected (B), or coinfected (C) *Gerbillus andersoni* rodents**.** The changes in rodent variables are indicated by increase ↑ and decrease ↓ arrows. Bart: *Bartonella* load in 1 µl of DNA extracted from rodent blood; IgG: *Bartonella*-specific antibody levels estimated by optical density units at 450 nm; TEMP: surface body temperature (°C); BM: body mass gain (g); FCM: fecal corticosterone metabolites (ng/g dry feces) used to assess stress levels; Var: trait variability; NAD: nocturnal activity duration (s/12 h); DAD: diurnal activity duration (s/12 h); NAF: nocturnal activity frequency (number of movements per 12 h); DAF: diurnal activity frequency (number of movements per 12 h); Co: control; Coin: coinfection; M: males.

* For male rodents only; ** Non-significant.

Fig. 2. Comparisons between the four treatment groups throughout the four infection periods.

Mean ± SE of *Bartonella* load (in 1 µl of DNA; A) and of rodent variable changes between the various infection periods (peak infection, sharp decrease in bacteremia, slow decrease in bacteremia, and *Mycoplasma* plateau) and the pre-inoculation period (differences between each measure at post-inoculation and pre-inoculation). Beside the changes in *Bartonella*-specific antibodies [IgG (450 nm); B] that show an insignificant trend, all other shown host variables include at least one significant between-group comparison. These are changes in body mass (g; C), surface body temperature (°C; D), physiological stress levels [measured by fecal corticosterone metabolites; FCM (ng/g dry feces); E], variability in body mass (g; F), variability in surface body temperature (°C; G), variability in log nocturnal activity duration (s/12 h; H), variability in diurnal activity duration (s/12 h; I), and variability in diurnal activity frequency (number of movement bouts per 12 h; J).

$^{*\nabla}$Significant between-group planned comparisons.

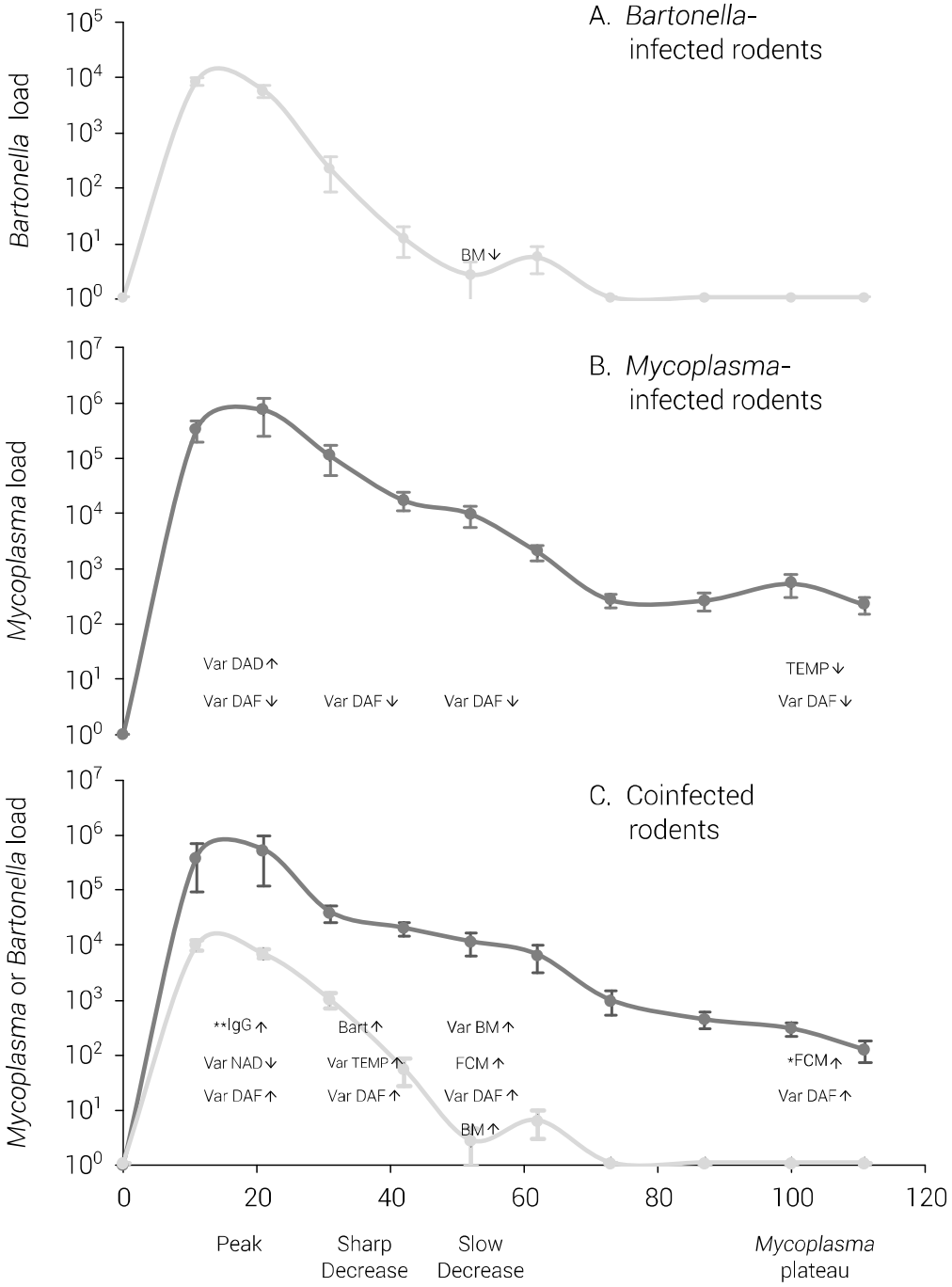
A. Bartonella-infected rodents
Bartonella load
BM↓
B. Mycoplasma-infected rodents
Mycoplasma load
Var DAD↑
Var DAF↓
Var DAF↓
Var DAF↓
TEMP↓
Var DAF↓
C. Coinfected rodents
Mycoplasma or Bartonella load
**IgG↑
Var NAD↓
Var DAF↑
Bart↑
Var TEMP↑
Var DAF↑
Var BM↑
FCM↑
Var DAF↑
BM↑
*FCM↑
Var DAF↑
0
20
40
60
80
100
120
Peak
Sharp Decrease
Slow Decrease
Mycoplasma plateau

Figure 1

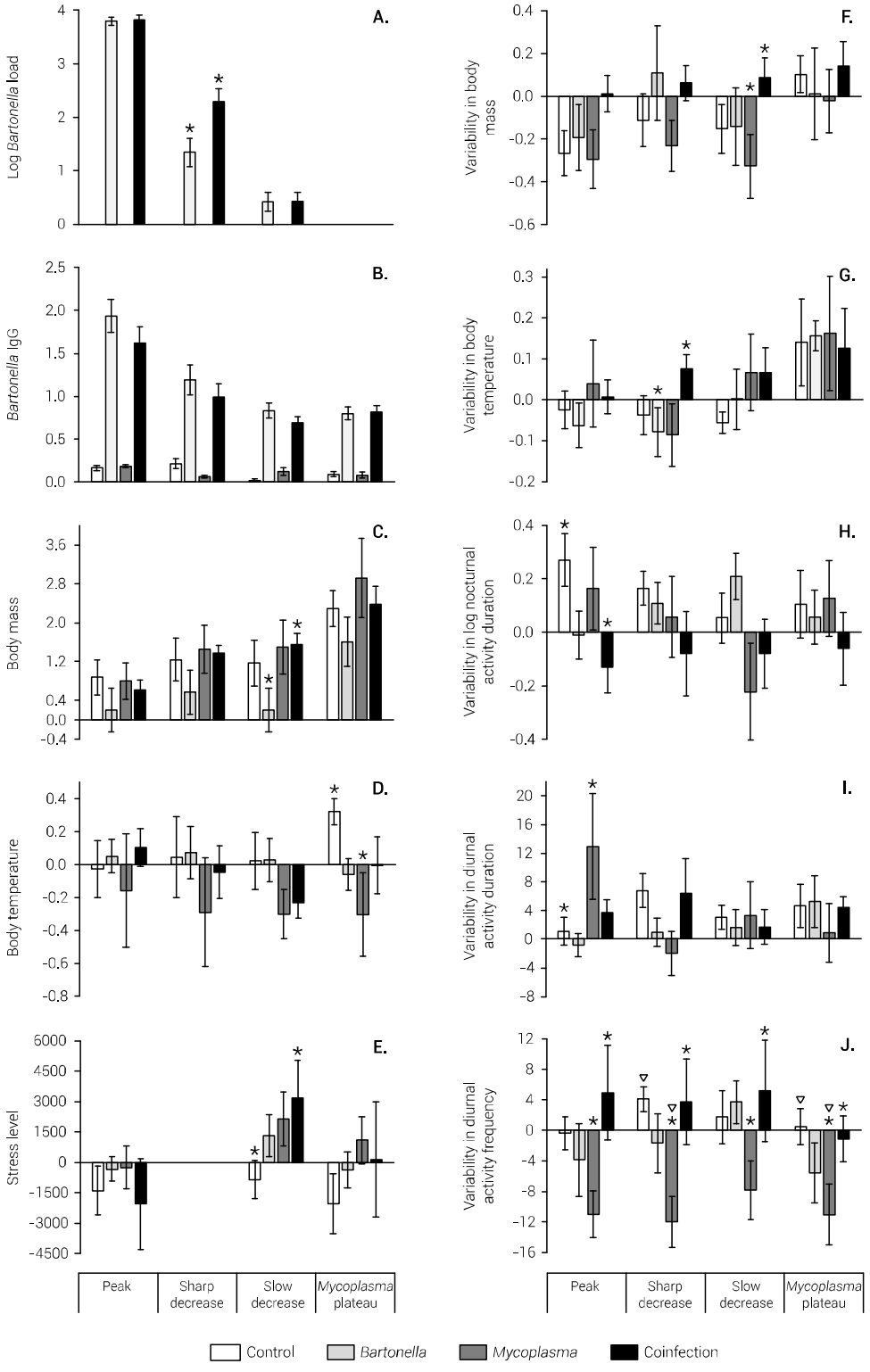
A.
Log Bartonella load
B.
Bartonella IgG
C.
Body mass
D.
Body temperature
E.
Stress level
F.
Variability in body mass
G.
Variability in body temperature
H.
Variability in log nocturnal activity duration
I.
Variability in diurnal activity duration
J.
Variability in diurnal activity frequency
Peak
Sharp decrease
Slow decrease
Mycoplasma plateau
Control
Bartonella
Mycoplasma
Coinfection

Figure 2